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270, 3216 (1995); H. C. Lee and R. Aarhus, *ibid.*, p. 2152.
15. H. C. Lee, *ibid.* 266, 2276 (1991); H. C. Lee, in *Ryanodine Receptors*, V. Sorrentino, Ed. (CRC Pharmacology and Toxicology Series, Boca Raton, FL, 1995), pp. 31–50; L. G. Mészáros, J. Bak, A. Chu, *Nature* 364, 76 (1993).
16. G. S. Prasad *et al.*, *Nature Struct. Biol.* 3, 957 (1996); H. C. Lee and R. Aarhus, *Cell Regul.* 2, 203 (1991).
17. G. J. Allen, S. R. Muir, D. Sanders, *Science* 268, 735 (1995).
18. S. R. Muir and D. Sanders, *FEBS Lett.* 395, 39 (1996).
19. Y. Wu, K. Hiratsuka, G. Neuhaus, N.-H. Chua, *Plant J.* 10, 1149 (1996); C. Bowler, G. Neuhaus, H. Yamagata, N.-H. Chua, *Cell* 77, 73 (1994); Erratum: *ibid.* 79, 743 (1994); G. Neuhaus, C. Bowler, R. Kern, N.-H. Chua, *ibid.* 73, 937 (1993); Erratum: *ibid.* 79, 743 (1994).
20. M. J. Terry and R. E. Kendrick, *J. Biol. Chem.* 271, 21681 (1996).
21. We constructed *kin2-GUS* by ligating 1358 base pairs (bp) (−1358 to +8) of *A. thaliana kin2* flanking 5′ sequence to a GUS reporter gene cassette [R. A. Jefferson *et al.*, *EMBO J.* 6, 3901 (1987)]. For *rd29A-GUS*, 1291 bp of *A. thaliana rd29A* 5′ flanking sequence (−1291 to +8) were used. The *rbcS-3C* polyadenylate addition sequence was placed at the 3′ end in both cases.
22. Y. C. Chang and L. L. Walling, *Plant Physiol.* 97, 1260 (1991).
23. Y. Wu, R. Foster, N.-H. Chua, unpublished data.
24. T. F. Walseth and H. C. Lee, *Biochim. Biophys. Acta* 1178, 235 (1993).
25. H. C. Lee and R. Aarhus, *ibid.* 1164, 68 (1993).
26. P. N. Benfey, L. Ren, N.-H. Chua, *EMBO J.* 8, 2195 (1989).
27. H. Kim, E. L. Jacobson, M. K. Jacobson, *Science* 261, 1330 (1993).
28. C. Schmidt, I. Schelle, Y.-J. Liao, J. I. Schroeder, *Proc. Natl. Acad. Sci. U.S.A.* 92, 9535 (1995).
29. C. Bowler, H. Yamagata, G. Neuhaus, N.-H. Chua, *Genes Dev.* 8, 2188 (1994).
30. Y. Lee *et al.*, *Plant Physiol.* 110, 987 (1996); E. V. Kearns and S. M. Assmann, *ibid.* 102, 711 (1993).
31. T. Hirayama, C. Ohto, T. Mizoguchi, K. Shinozaki, *Proc. Natl. Acad. Sci. U.S.A.* 92, 3903 (1995).
32. We constructed the *kin2-LUC* transgene by fusing *Arabidopsis kin2* 5′ upstream sequence (−1358 to −1) to a firefly *LUC* cassette that includes 67 bp of the tobacco mosaic virus 5′ untranslated sequence and the pea *rbcS-E9* poly(A) addition sequence.
33. A. J. Millar, S. R. Short, N.-H. Chua, S. A. Kay, *Plant Cell* 4, 1075 (1992).
34. We measured Ca^{2+} release using a sea urchin microsomal assay. Microsomes were loaded with Ca^{2+}, and Ca^{2+} release from the purified extracts and standard compounds was measured by fluorimetry (*12*). Two peaks of Ca^{2+}-release activity from the last purification step were detected; one was primarily desensitized to cADPR, and the other to $NAADP^+$ (*14*). Neither peak exhibited desensitization to IP_3. Desensitization of channel activity has previously been used as a method to determine the qualitative presence of molecules [T. F. Walseth, R. Aarhus, R. J. Zeleznikar, Jr., H. C. Lee, *Biochim. Biophys. Acta* 1094, 113 (1991)]. In addition, release activity from the first peak (that is, "cADPR" desensitized) was potentiated by caffeine, an agonist of cADPR activity, and inhibited (50 to 90%) by 8-NH_2-cADPR (0.2 μM in assay), an antagonist. Ca^{2+} release from the peak that was desensitized to cADPR was monitored over the time course and in three separate experiments (Fig. 1B). Ca^{2+} release consistently exhibited two maxima: one at 1 hour and the other at 4 hours after ABA treatment. Northern (RNA) blotting experiments were conducted essentially as described [S. A. Barnes *et al.*, *Plant Cell* 8, 601 (1996)] with a *kin2* probe specific for the 3′ untranslated region (*2*).
35. W. Masuda *et al.*, *FEBS Lett.* 405, 104 (1997).
36. M. L. W. Knetsch, M. Wang, B. E. Snaar-Jagalska, S. Heimovaara-Dijkstra, *Plant Cell* 8, 1061 (1996).
37. We thank S. Cahill for advice on Ca^{2+} fluorimetry, D. Cowburn for use of his facilities, and R. F. Irvine for IP_3. Supported by NIH grant HD17484 (to H.C.L.) and U.S. Department of Energy (DOE) grant DE-FG02-84 ER20142 (to N.-H.C.). J.K. was supported by a Life Science Research Foundation–DOE Biosciences postdoctoral fellowship (5-29329), and E.M. was supported by a Human Frontier Science Program fellowship.

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Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts

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Ovine primary fetal fibroblasts were cotransfected with a neomycin resistance marker gene (*neo*) and a human coagulation factor IX genomic construct designed for expression of the encoded protein in sheep milk. Two cloned transfectants and a population of neomycin (G418)–resistant cells were used as donors for nuclear transfer to enucleated oocytes. Six transgenic lambs were liveborn: Three produced from cloned cells contained factor IX and *neo* transgenes, whereas three produced from the uncloned population contained the marker gene only. Somatic cells can therefore be subjected to genetic manipulation in vitro and produce viable animals by nuclear transfer. Production of transgenic sheep by nuclear transfer requires fewer than half the animals needed for pronuclear microinjection.

Microinjection of DNA into the pronuclei of fertilized oocytes has been the only practical means of producing transgenic livestock since the method was established in 1985 (*1*). However, only a small proportion (~5%) of animals integrate the transgene DNA into their genome (*2*, *3*). In addition, because the timing and site of integration are random, many transgenic lines do not provide sufficiently high levels of transgene expression or germline transmission. The consequent inefficient use of animals and associated high costs are a major drawback to pronuclear microinjection.

In mice, embryonic stem cells provide an alternative to pronuclear microinjection as a means of transferring exogenous DNA to the germline of an animal and allow precise genetic modifications by gene targeting (*4*, *5*). However, despite considerable efforts, embryonic stem cells capable of contributing to the germline of any livestock species have not been isolated (*6–11*).

Recently, viable sheep have been produced by transfer of nuclei from a variety of somatic cell types cultured in vitro (*12–14*). We now demonstrate that nuclear transfer from stably transfected somatic cells provides a cell-mediated method for producing transgenic livestock.

We have used a transgene designed to express human clotting factor IX (FIX) protein in the milk of sheep. FIX plays an essential role in blood coagulation, and its deficiency results in hemophilia B (*15*). This disease is currently treated with FIX derived mainly from human plasma. Recombinant FIX produced in milk would provide an alternative source at lower cost and free of the potential infectious risks associated with products derived from human blood.

The transgene construct, pMIX1 (*16*), comprises the human FIX gene, containing the entire coding region (*17*), linked to the ovine β-lactoglobulin (BLG) gene promoter, which has been previously shown to provide a high level of transgene expression in ovine mammary glands (*18*). Analysis of pMIX1 expression in transgenic mice showed that seven of seven female founders expressed FIX in their milk (*19*). The level of expression in two animals (125 μg/ml) exceeded that achieved in previous studies (*20*, *21*), indicating that pMIX1 is functional and suitable for introduction into sheep.

Primary strains of ovine cells, termed PDFF (Poll Dorset fetal fibroblast) 1 to 7, were derived from seven day-35 fetuses from the specific pathogen-free flock at PPL Therapeutics (*22*). Sex analysis of each cell strain by the polymerase chain reaction (PCR) (*23*) revealed PDFF5 to be male and

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Table 1. Results of nuclear transfer. Nuclear transfer was performed as described previously (*12, 13*). All cells were exposed to a reduced serum concentration (0.5%) for 5 days before use as nuclear donors. PDFF5 cells were used for nuclear transfer at passage 2 or 3, PDFF2 transfected pools at passage 5 to 7, and transfected clones PDFF2-12 and PDFF-13 at passage 7 to 9. Liveborn lambs were defined as those with a heartbeat and able to breathe unassisted at birth.

Measurement	PDFF5 (non-transfected)	PDFF2 pool	PDFF2-12	PDFF2-13
Reconstructed embryos	82	224	89	112
No. developed to morulae or blastocysts	5 (6.1%)	22 (9.8%)	19 (21.4%)	23 (20.5%)
Embryos transferred	5	22	19	21
Recipients	2	9	7	6
Pregnancies at day 60	2	4	4	1
Fetuses at day 60 (% of embryos transferred)	3 (60%)	4 (18.2%)	6 (31.6%)	1 (4.8%)
Liveborn lambs (% of embryos transferred)	1 (20%)	3 (13.6%)	2 (10.5%)	1 (4.8%)
Nuclear transfer efficiency (% live lambs from reconstructed embryos)	1.22%	1.34%	2.25%	0.89%

the other six to be female.

Trial experiments indicated that both PDFF2 and PDFF5 cells could be readily transfected with a *lacZ* reporter gene with the use of the cationic lipid reagent Lipofectamine. PDFF2 cells at passage 1, after 3 days in culture, were cotransfected with pMIX1 DNA and the selectable marker construct PGKneo, and stable transfectants were selected with G418. Because the effects of drug selection and growth as single-cell clones on the ability of cells to support nuclear transfer were unknown, cells were then treated in two ways: One group was grown at high density under G418 selection and then cryopreserved as a pool for nuclear transfer. The other group was plated at low density under G418 selection, and cloned transfectants were grown from isolated colonies (*22*). A total of 24 clones was isolated, of which 21 were expanded for analysis of genomic DNA. Ten clones were found to contain pMIX1 by DNA hybridization analysis (*24*).

Untransfected PDFF2 cells cultured to passage 19 over a period of 80 days exhibited a modal chromosome number of 54, the euploid ovine chromosomal complement. The chromosome number of the four most rapidly growing pMIX1-transfected clones (PDFF2-12, -13, -31, -38) was determined at passage 6 or 7, after an average of 40 days in culture, and that of the uncloned PDFF2 pool was determined at passage 5, after 19 days in culture. Each clone and the pool showed a modal chromosome number of 54, indicating the absence of gross chromosomal instability during culture and drug selection.

We have proposed that induction of quiescence in nuclear donor cells by serum deprivation is necessary for successful nuclear transfer (*12*). After 5 days of culture in medium with a reduced serum content (0.5%), immunofluorescence detection of proliferating-cell nuclear antigen (PCNA), which is an indicator of active DNA replication, showed that none of the cells analyzed was in S phase, consistent with cell cycle arrest (*25*). Restoration of serum content to 10% reversed this effect and cell growth resumed.

Four cell types were used as nuclear donors: untransfected male PDFF5 cells, pooled female PDFF2 transfectants, and two transfected clones, PDFF2-12 and PDFF2-13, which contained >10 and ~5 copies of the pMIX1 transgene, respectively. Transfer of nuclei from each cell type into enucleated oocytes derived from Scottish Blackface ewes was performed as previously described (*12, 13*).

Live lambs were obtained from all four cell types (Table 1). As expected, animals derived from PDFF5 cells were male and those from PDFF2 cells were female. The efficiency of nuclear transfer, expressed as the number of liveborn lambs obtained per 100 reconstructed embryos, varied from 0.89% for PDFF2-13 to 2.25% for PDFF2-12. This efficiency is similar to the value (1.35%) that we obtained previously for nonmanipulated fetal fibroblasts from another breed of sheep (BLWF1) (*13*).

Pregnancies resulting from embryo trans-

Table 2. Characteristics of nuclear transfer–derived lambs. Outcomes of 11 pregnancies resulting from nuclear transfer of PDFF donor cells. When judged necessary, labor was induced by injection of dexamethasone at day 153 of gestation; when required, cesarean section (CS) was performed 24 to 52 hours later. The average duration of gestation for the Poll Dorset flock at PPL Therapeutics is 145 days.

Pregnancy no.	Nuclear transfer donor cell type	Lamb	Gestation (days)	Birth weight (kg)	*neo*	FIX	Sex	Comments
1	PDFF5	7LL5	147	3.8			M	Unassisted birth
2 (twins)	PDFF5	7LL6*	150	3.4			M	Stillbirth, one fetus dead for ≤1 week
		7LL7*	150	3.7			M	
3	PDFF2 pool		<80					Regressed
4	PDFF2 pool	7LL8	155	7.6	(+)	(−)	F	Assisted birth because of position of lamb
5	PDFF2 pool	7LL9*	161	6.3	(+)	(−)	F	Induced, CS 52 hours later, died 90 min postpartum, meconium in lung
6	PDFF2 pool	7LL12	155	8.7	(+)	(−)	F	Induced, CS 52 hours later
7	PDFF2-12	7LL3*	130				F	Spontaneous abortion
8 (twins)	PDFF2-12	7LL10*	132	3.6	(+)	(+)	F	Loss of fetal heartbeat, induced, CS,
		7LL11*	132	4.5	(+)	(+)	F	stillbirth, one fetus abnormal
9	PDFF2-12	7LL14*	148	3.6	(+)	(+)	F	Induced, CS 24 hours later, heartbeat, no breathing
10 (twins)	PDFF2-12	7LL15	155	4.6	(+)	(+)	F	Induced, unassisted birth, 7LL16
		7LL16*	155	3.0	(+)	(+)	F	euthanized at 14 days, heart defect
11	PDFF2-13	7LL13	155	5.5	(+)	(+)	F	Induced, unassisted birth

*Lamb died or was euthanized for animal welfare reasons.

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fer were determined by ultrasound scan at about 60 days after estrus, and development was subsequently monitored at regular intervals. Of the original 14 fetuses, 7 were liveborn, as defined by heartbeat and unassisted breathing (Table 2). Postmortem examination of aborted fetuses and dead lambs did not reveal any common factor as a cause of death.

All animals derived from PDFF cells exhibited a prolonged gestation, and, with the exception of animals 7LL5 to 7LL8, labor was induced artificially. Delayed delivery was likely the cause of death of lamb 7LL9. Subsequently, all surrogate ewes were induced at day 153, and, if necessary, cesarean section was performed. Three of 11 pregnancies were twin pregnancies. In two instances (7LL6 and 7LL7 and 7LL10 and 7LL11), the death of one fetus in late pregnancy probably resulted in the death of the sibling.

The birth weight of nuclear transfer–derived lambs whose gestation exceeded 145 days ranged from 3.0 to 8.7 kg, with a mean of 3.7 kg for twins and 5.9 kg for single pregnancies. Poll Dorset lambs in the PPL Therapeutics New Zealand–derived flock have mean weights of 3.75 kg for twins and 5.1 kg for single pregnancies. However, comparison is complicated by the fact that nuclear transfer–derived lambs were gestated in Scottish Blackface surrogate mothers. All animals from PDFF2 cells had an undershot lower jaw that did not interfere with their well-being. This characteristic is a genetic trait that occurs sporadically in the Poll Dorset breed and is considered to be unrelated to nuclear transfer. The PDFF5 lambs did not show this feature.

DNA from nuclear transfer–derived lambs was analyzed for the presence of pMIX1 and PGKneo transgenes (Fig. 1). All fetuses and animals derived from the transfected PDFF2 cells were transgenic. The three animals derived from the PDFF2 pool (7LL8, -9, -12) contained the selectable marker gene but lacked the FIX transgene (Fig. 1, A and B). Fetuses and lambs derived from the cell clones PDFF2-12 (7LL10, -14, -15, -16) and PDFF2-13 (7LL13) contained both the FIX transgene (Fig. 1B) and PGKneo.

Our approach has shown that cell-mediated transgenesis is possible in a mammal other than the mouse. The technique is still in the early stages of development and problems remain to be addressed—in particular, the lack of spontaneous parturition and the incidence of perinatal mortality. However, the mortality rate we observed (46%) was exacerbated by two twin pregnancies in which the death of one lamb in late gestation may have resulted in the loss of the other. The mortality rate for nontwin pregnancies was 28.6%, higher than that occurring after normal breeding (~8%) but similar to that observed after nuclear transfer with embryonic blastomeres (5 to 40%) (*26*). Our data therefore do not suggest any correlation between lamb mortality and extended culture or genetic manipulation of the donor cell. Many types of manipulation of preimplantation embryos—for example, in vitro oocyte maturation and fertilization, in vitro culture, asynchronous embryo transfer, and progesterone treatment of the mother—have been shown to increase fetal morbidity and mortality (*26*, *27*). An increased understanding of the interaction between the transplanted nucleus and the host cytoplasm and the relation between the early embryo and the maternal environment, together with improved culture systems, should increase the success of embryo production and manipulation in vitro.

The use of somatic cell donors for nuclear transfer in livestock offers many advantages over pronuclear microinjection. Since 1989, PPL Therapeutics has generated a substantial number of transgenic sheep by pronuclear microinjection. A total of 51.4 animals are required to produce one transgenic lamb by pronuclear microinjection, compared with 20.8 animals in the present study by nuclear transfer, values that differ by a factor of ~2.5 (Table 3). The most important difference is that no recipients are wasted gestating nontransgenic lambs in the nuclear transfer technique.

Gestation of large numbers of nontransgenic embryos represents a major source of inefficiency (*28*). Several schemes have been devised to identify transgenic embryos before embryo transfer, either by detection of the transgene in embryo biopsies by PCR (*29*) or by co-expression of a marker gene (*30*, *31*). However, these methods, with the possible exception of that of Takada *et al.* (*30*), are restricted by the persistence of

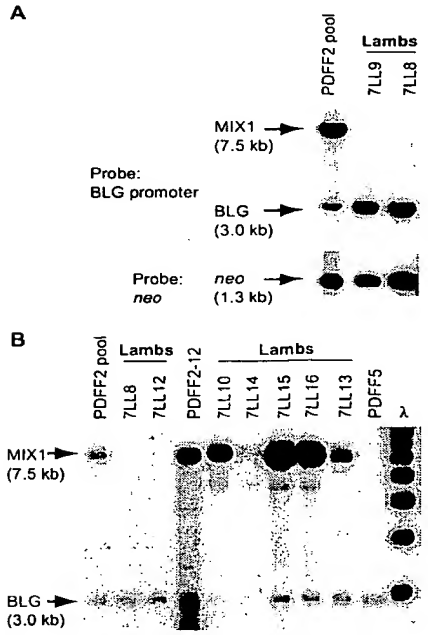

Fig. 1. DNA analysis of transfected clones and transgenic sheep. Genomic DNA was isolated from the blood of live animals or tongue samples from dead animals, digested with Bam HI and Eco RI, and subjected to Southern hybridization with either a 1.8-kb fragment of the BLG promoter or the *neo* gene. (**A**) Southern analysis of the uncloned pool of cells (PDFF2 pool), and two lambs (7LL8 and 7LL9) derived from them, for the presence of pMIX1 and PGKneo. (**B**) Assay for the presence of the pMIX1 transgene in lambs derived from the PDFF2 pool (7LL8 and 7LL12) and from the transfected clones PDFF2-12 (7LL10, 7LL14 to 7LL16) and PDFF2-13 (7LL13). PDFF5 cells were not transfected. The positions and sizes of fragments corresponding to the transgenes and the endogenous BLG gene are indicated. The lane marked λ is a 1-kb ladder of phage λ fragments from 3 to 12 kb.

Table 3. Comparison of the production of transgenic sheep by nuclear transfer or pronuclear microinjection.

Parameter	Pronuclear microinjection (1989–1996)	Nuclear transfer of PDFF2 transfectants
Oocyte donors	982	68
Intermediate recipients*	Not applicable	14
Final recipients	1895	22
Total number of sheep used	2877	104
Established pregnancies (% of final recipients)	912 (48%)	9 (41%)
Lambs born	1286	6
Viable transgenic lambs born†	56	5
Percentage of offspring transgenic	4.35%	100%
Sheep required for production of one transgenic lamb	51.4	20.8

*After nuclear transfer, intermediate recipients are used to allow development of reconstructed embryos to blastocyst stage. †Defined as those alive at 1 week of age.

unintegrated DNA during the short time that embryos can be cultured before embryo transfer. In contrast, cells transfected in vitro can be analyzed extensively before effort is devoted to large animals. This advantage will be particularly important in instances in which microinjection is inefficient; for example, with large constructs such as yeast artificial chromosomes.

Delayed integration of microinjected DNA into the embryo genome often results in mosaic founder animals. The reduced rate of transgene transmission resulting from germline mosaicism can hinder or prevent the establishment of transgenic lines from potentially valuable founder animals. In contrast, animals produced by nuclear transfer are entirely transgenic.

Nuclear transfer allows the sex of transgenic animals to be predetermined and thus offers a further twofold increase in efficiency relative to pronuclear microinjection when the sex of the transgenic founder animal is critical. If, for example, the primary interest is the expression of human proteins in milk, the founder generation can be all females. Sheep with different random integrations of the transgene can be produced by nuclear transfer from independent cell clones and the milk analyzed. After a suitable clone has been identified, the corresponding stock of cells can be used to generate an "instant flock" by further nuclear transfer. Such a flock could be superior to those produced by conventional breeding as a source of proteins for human therapy because genetic identity would contribute to the consistency of the medicinal product.

The procedures of transfection, drug selection, and growth from single-cell clones described here are essentially the same as those required for gene targeting. The realistic prospect of targeted genetic manipulation in a livestock species should open a vast range of new applications and research possibilities.

REFERENCES AND NOTES

1. R. E. Hammer *et al.*, *Nature* **315**, 680 (1985).
2. W. H. Eyestone, *Reprod. Fert. Dev.* **6**, 647 (1994).
3. S. Damak, H. Su, N. P. Jay, D. W. Bullock, *Bio/Technology* **14**, 185 (1996).
4. R. Ramirez-Solis and A. Bradley, *Curr. Opin. Biol.* **5**, 528 (1994).
5. E. P. Brandon, R. L. Idzerda, G. S. McKnight, *Curr. Biol.* **5**, 625 (1995); *ibid.*, p. 758; *ibid.*, p. 873.
6. E. Notarianni, C. Galli, S. Laurie, R. M. Moore, M. J. Evans, *J. Reprod. Fertil.* **43**, 255 (1991).
7. M. B. Wheeler, *Reprod. Fertil. Dev.* **6**, 563 (1994).
8. R. W. Gerfen and M. B. Wheeler, *Animal Biotechnol.* **6**, 1 (1995).
9. S. L. Stice, N. S. Strelchenko, C. L. Keefer, L. Mathew, *Biol. Reprod.* **54**, 100 (1996).
10. H. Shim, A. Gutierrez-Adan, L. R. Chen, R. H. Bon-Durant, G. B. Anderson, *Theriogenology* **47**, 245 (1997).
11. J. B. Cibelli *et al.*, *ibid.*, p. 241.
12. K. H. S. Campbell, J. McWhir, W. A. Ritchie, I. Wilmut, *Nature* **380**, 64 (1996).
13. I. Wilmut, A. E. Schnieke, J. McWhir, A. J. Kind, K. H. S. Campbell, *ibid.* **385**, 810 (1997).
14. D. N. Wells, P. M. Misica, A. M. Day, H. R. Tervit, *Biol. Reprod.* **57**, 385 (1997).
15. G. G. Brownlee, *Biochem. Soc. Trans.* **15**, 1 (1987).
16. For construction of pMIX1, human FIX DNA from an Nhe I site at position 2982 to a Bst BI site at position 33,840 (positions refer to EMBL database file HSFIXG) was derived from overlapping genomic λ clones. A 3′ fragment from the Bst BI site to position 34,346 was derived by PCR amplification and a Xho I site introduced. The 31.36-kb fragment containing the FIX coding region was assembled in Bluescript KS− vector (Stratagene) that had been digested with Xba I and Xho I. The entire FIX gene was then excised as a Not I–Xho I fragment and inserted into the ovine BLG expression vector pMADS+ that had been digested with Not I and Xho I. The pMADS+ vector consists of 4.2 kb of BLG promoter sequence, 30 bp of 5′ untranscribed region, a multicloning site (Eco RV, Not I, Bgl II, Nhe I, Xho I, Cla I), 170 bp of 3′ untranscribed region including a polyadenylate addition site, and 2 kb of 3′ flanking region cloned into pUC18, containing a modified polylinker to allow excision of the insert with either Mlu I or Pvu I.
17. S. Yoshitake, B. G. Schach, D. C. Foster, E. W. Davie, K. Kurachi, *Biochemistry* **24**, 3736 (1985).
18. A. S. Carver *et al.*, *Bio/Technology* **11**, 1263 (1993).
19. Transgenic mice were produced by microinjection of a 37.8-kb Mlu I fragment of pMIX1 into the male pronucleus of fertilized mouse oocytes. Milk was collected from transgenic founder females at day 10 postpartum. FIX protein in milk was assayed by enzyme-linked immunosorbent assay with polyclonal rabbit antiserum to human FIX (Dako) and quantified by comparison with purified human FIX standard (Diagnostica Stago).
20. F. Yull *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10899 (1995).
21. S. Jallat *et al.*, *EMBO J.* **9**, 3295 (1990).
22. PDFF cell strains were isolated by disaggregation of day-35 fetuses and cultured as described previously (*13*). At first passage, 2 × 10^5 PDFF2 cells were plated into a 3.5-cm-diameter well and cotransfected with 0.5 μg of PGKneo and 1.5 μg of Mlu I–digested pMIX1 DNA with the use of Lipofectamine (Gibco). Forty-eight hours after transfection, cells were split 1:10 and G418 added to a final concentration of 0.6 mg/ml. Transfected PDFF2 cells achieved subconfluence after selection for 6 days. At the third passage, one portion of the cells was split 1:10 and then subjected to selection for an additional 5 days before cryopreservation as an uncloned population. Other portions were split 1:1000 or 1:5000 and subjected to G418 selection for an additional 7 days. Individual colonies were isolated and expanded for cryopreservation at passage 5, and a portion of each clone was grown further for chromosome counting and Southern (DNA) blot analysis.
23. The sex of strains PDFF1 to PDFF7 was determined by PCR, essentially as described [R. Griffiths and B. Tiwari, *Mol. Ecol.* **2**, 405 (1993); B. W. Kirkpatrick and R. L. Monson, *J. Reprod. Fert.* **98**, 335 (1993)].
24. Genomic DNA isolated from cloned pMIX1 transfectants was digested with Bam HI and Eco RI and subjected to Southern blot analysis with a 1.8-kb fragment of the BLG promoter.
25. PCNA analysis of serum-deprived cells was performed essentially as described [I. R. Kill *et al.*, *J. Cell Sci.* **100**, 869 (1991)].
26. Th. A. M. Kruip and J. H. G. den Daas, *Theriogenology* **47**, 43 (1997).
27. S. K. Walker, K. M. Hartwich, R. F. Seamark, *ibid.* **45**, 111 (1996).
28. R. J. Wall and G. E. Seidel, *ibid.* **38**, 337 (1992).
29. R. A. Bowen *et al.*, *Biol. Reprod.* **50**, 664 (1994).
30. T. Takada *et al.*, *Nature Biotechnol.* **15**, 458 (1997).
31. E. M. Thompson, P. Adenot, F. I. Tsuji, J. P. Renard, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1317 (1995).

32. We thank J. Bracken and M. Malcolm-Smith for technical assistance in large animal work, H. Bowran and D. McGavin for care of the animals, D. Cottom for providing data on microinjection studies, the Animal Sciences Team at PPL Therapeutics for help in deriving the PDFF cells, Y. Gibson and the Small Animal Unit at PPL Therapeutics for generating FIX transgenic mice, S. Bruce for FIX protein analysis, G. G. Brownlee for human FIX genomic λ clones, D. Melton for PGKneo, A. R. Scott for technical assistance with molecular biology, and I. Garner for constructive discussions during the course of the project. The experiments were conducted under the Animals (Scientific Procedures) Act 1986 and with the approval of the Roslin Institute Animal Welfare and Experiments Committee.

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